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磷酸盐饥饿时番茄幼苗根部质膜蛋白组分的变化*

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摘要：对处于磷酸盐饥饿条件下的番茄幼苗根部质膜以及去除质膜的其他膜部分的蛋白质含量及组分的变化进行了检测。结果显示，磷酸盐饥饿第 7 d 时，受胁迫苗根部质膜及去除质膜的其他膜蛋白质含量与各自的对照相当。而 SDS－PAGE 结果表明，磷酸盐饥饿第 7 d 时受胁迫苗根部质膜蛋白质中出现 4 条对照中所没有的新的多肽（分子量分别为 34 kD，36 kD，46 kD 和 49 kD）。该结果经浓度梯度电泳得到进一步的证实。本文推测在受胁迫苗根部质膜中出现的这些新的多肽可能是磷酸盐饥饿诱导蛋白并作为磷酸盐饥饿救援系统的组分。但在去除质膜的其他膜蛋白质中未看到胁迫处理与对照之间多肽表达的明显的差别。

关键词：番茄；磷酸盐饥饿；救援系统；诱导蛋白

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Changes of Protein Components of Plasma Membranes from the Roots of Tomato Seedlings under Phosphate Starvation*

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Abstract：The changes of protein components of plasma membranes and plasma－membrane－depleted vesicles from the roots of tomato seedlings under phosphate starvation were studied in this paper. Firstly，the results revealed that，under phosphate starvation for 7 days，the contents of proteins of plasma membranes and plasma－membrane－depleted vesicles from the roots of tomato seedlings approximated to those of their unstressed controls respectively. Secondly，the results from SDS－PAGE of proteins showed that there were four novel polypeptides（mw：34 kD，36 kD，46 kD，49 kD）which existed in the plasma membranes from the roots of tomato seedlings under phosphate starvation for 7 days but not in those of controls. The above－mentioned results of SDS－PAGE were further confirmed by CG－PAGE. It was supposed that these new

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Abbreviations：CG：concentration gradient；DTT：dithiothreito；EDTA：ethylenediamine tetraacetate；Mes：2－N－morpholino ethane sulfonic acid；mw：molecular weight；PAGE：polyacrylamide gel electrophoresis；PEG：polyethyleneglycol；PVP：polyvinylpyrrolidone；SDS：sodium dodecyl sulfate；Tris：trihydroxymethyl aminomethane

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polypeptides might be specifically induced by phosphate starvation and as the components of phosphate starvation rescue systems. However, the results in this study did not find the existance of differential expression of proteins of plasma – membrane – depleted vesicles in the stressed seedlings and their controls.

Key words: Tomato (*Lycopersicon esculentum*); Phosphate starvation; Rescue system; Inducible proteins;

Phosphorus is a very important element in plant nutrition and orthophosphate anion (Pi, i. e., $H_2PO_4^-$ or HPO_4^{2-}) is the preferential form of this element absorbed by plants. However, Pi is one of the least available mineral nutrients in many environments, which often lead to phosphate starvation in plants (Clarkson *et al*, 1991). The principal goal of plants responded to Pi – starvation is to improue their capacity for absorbing and utilizing Pi that may be available (Fife *et al*, 1990). It has been found that Pi – starvation induced high affinity transport systems in both bacteria and fungi and higher plants (Lee 1982; Lefebvre *et al*, 1982; Cogliatti *et al*, 1983; Takashi *et al*, 1992), but there have been few attempts to identify the molecular species responsible for secondary or coupled phosphate transport across the plant plasma membrane. It has been well known that identification of protein components is an initial step for the elucidation of the molecular mechanisms and the regulations of these transport processes. Analyzing the differential expression of proteins in response to specific nutrient stress is a good way of identifying specifically synthesized polypeptides under the specific stress (Sussman *et al*, 1989; Clarkson *et al*, 1989a, 1989b; Ruiz – Cristin *et al*, 1991). For example, several polypeptides in the plasma membrane fraction have been identified during nitrate transport induction (Dhugga *et al*, 1988). In fact, adaptations of plants to almost all environmental stress have involved the production of novel proteins (Sachs *et al*, 1986; Xu *et al*, 1991).

This paper studied the possible changes of protein components of plasma membranes from the roots of tomato seedlings during their adaptation to phosphate starvation and attempted to identify the specific phosphate – starvation – induced polypeptides responsible for phosphate transport. In addition, the protein components of plasma – membrane – depleted vesicles from the roots of tomato seedlings were also analyzed in this paper in order to find possible changes of protein expression in the fractions in response to Pi – starvation.

Materials and Methods

Growth of plants Seeds of tomato (*Lycopersicon esculentum* Mill. Yangza No. 1) were washed clean with water and then surface – sterilized with antiformin for 0.5 h, then washed with water again for 1 h and with distilled water for several times, finally sowed on distilled – water – soaked filter papers in culture dishes and germinated at 26℃ in darkness. Three days later, the seedlings were arranged to grow in complete and Pi – depleted nutrient solutions respectively at 26℃ with 12 h day – time illumination of 96 $W \cdot m^{-2}$. All the two nutrient solutions contained the following chemicals: 5 mmol / L $Ca(NO_3)_2$, 5 mmol / L KNO_3, 2 mmol / L $MgSO_4$, Fe – EDTA and trace elements, except that Pi – depleted nutrient solution substituted 1 mmol / L KCl for 1 mmol / L KH_2PO_4 which were contained in complete nutrient solution. The nutrient solutions were adjusted to pH 5.5, renewed once a week and supplemented with distilled water timely during the culture period.

Membrane extraction and fractionation Membranes were prepared according to the method of two – phase partition technique (Hu *et al*, 1993). Three to five grams (fresh weight) of roots were homogenized in 60 mL of cold buffer (consisting of 1.0 mmol / L EDTA, 3.0mmol / L DTT, 0.5mmol / L sucrose, 0.6% (w/w) PVP, 15.0mmol / L Tris – HCl (pH 7.8) at 4℃ (All the following operations were carried out at 4℃) for 2 ~ 3 min. The homogenate was filted through a nylon mesh (240 μm style), then the filtrate was centrifuged at 13000 × g for 15 min and the pellet was discarded. The supernatant was centrifuged at 105000 × g for 30 min and the pellet was suspended in 3 mL of phosphate diluting buffer (consisting of 0.25mol / L sucrose and 5 mmol / L KH_2PO_4, pH 7.8) as the crude membrane fractions. The crude membrane fractions was mixed vigorously with the two phase systems (consisting of 62% (w/w) Dextran T 500, 62% (w/w) PEG 4000, 5.0mmol / L KCl, 8.5% (w/w) sucrose, 4 mmol / L KH_2PO_4, pH 7.8) in proportion of 1∶3. The mixture was separated into two (upper and lower) phases after centrifuging at 2000 × g for 10 min. The two phases were respectively mixed with two phase systems and centrifuged again. The supernatants from upper phases were collected and diluted three folds with diluting buffer (containing 0.25 mol / L sucrose, 0.1 mmol / L DTT, 5.0mmol / L Tris – Mes, pH 7.2) and then centrifuged at 105000 × g for 30 min. The pellet was suspended in 1.0 mmol/ L diluting buffer and used as purified plasma – membrane – enriched fraction. The lower phases were also collected, diluted, centrifuged and suspended as the upper phase did and used as plasma – membrane – depleted vesicles fraction.

Sample preparation for PAGE Samples for PAGE were prepared with reference to the method as Hurkman and Tanaka (1986) described. Membrane samples (0.5 mL) were mixed vigorously with 0.5 ml extraction buffer (0.1 mol / L Tris, 0.05 mol / L NaEDTA, 0.1 mol / L KCl, 2% (v/v) (– mercaptoethanol and 6 mmol / L HCl, pH 8.4). After 10 min incubation on ice, the mixture was separated into two phases at 11600 × g for 10 min. The aqueous phase was discarded without disturbing the protein precipitated at the interface. The extraction buffer was added to the pellet once more and centrifuge at 11600 × g for 10 min was repeated. Then the pellet was mixed with 0.5 mL ammonium acetate (0.1 mol / L) in ice – cold methanol and incubated overnight at – 20℃. After centrifuge at 11600 × g for 10 min, the pellet was washed twice with fresh methanol containing 0.1 mol / L ammonium acetate and finally with 80% (v/v) acetone and then partially dried with N_2 and resuspended in SDS – PAGE sample buffer (2% (w/v) SDS, 10% (w/v) glycerol, 0.02 mol / L DTT, 0.06 mol / L Tris – HCl and 0.05% (w/v) bromophenol blue, pH 6.8).

SDS – PAGE Gels of 0.8 mm thickness and 16 cm length were prepared containing 12% (w/v) Acrylamide, 0.1% (w/v) N, N' – Methylene bisacylamide and 375 mmol / L Tris – HCl (pH8.8), with a 4% (w/v) acylamide, 0.03% (w/v) N, N' – methylene bisacrylamide and 60 mmol / L Tris – HCl (pH6.8) stacking gel of 1.5 cm length. In addition, gels of concentration gradient (CG) were prepared according to the method described by Guo (1991) using the gradient mixer produced by Pharmacia LKB. Electrode buffer contained 25 mmol / L Tris, 190 mmol / L glycine (pH 8.3) and 0.1% (w/v) SDS. Gels were run at 240 V for 6 h. The standard proteins were products of the Dongfeng Biochemical Regent Factory in China. The molecular weights of proteins were determined according to the linear regression equation obtained by the migration rate of the standard proteins （value of X coordinate axis） and the logarithm of molecular weight of the standard proteins （value of Y coordinate axis）.

Silver staining of gels Gels were silver – stained according to the method described by Blum *et al* （1987）. Gels were fixed with 50% methanol， 12% acetic acid and 0.5mL / L 37% formaldehyde for 1 h or more， then washed with 50% ethanol for 3 × 20 min. After pretreated with 0.2 g / L $Na_2S_2O_3 \cdot 5H_2O$ for exactly 1 min， the gels were rinsed with distilled water （d H_2O） for 3 × 20 seconds and then impregnated with 2 g / L $AgNO_3$， 0.75 ml / L 37% formaldehyde for 20 min. The gels were rinsed with d H_2O again for exactly 2 × 20 seconds and then developed with 60 g / L Na_2CO_3， 4 mg / L $Na_2S_2O_3 \cdot 5H_2O$ and 0.5 mL / L formaldehyde for 10 min. The gels were washed with d H_2O for 2 × 2 min and the staining of the gels was stopped with the treatment of 50% methanol and 12% acetic acid. Finally the gels were washed with 50% methanol for more than 20 min and kept with 50% methanol at 4℃.

Scaning of gels Gels were scanned with a scanner of Shimadzu CS – 930 style at 450 nm of wave length.

Protein content determination Protein contents of membrane fractions were determined through the method of Coomassie brilliant blue G – 250 （1980）.

Results

Contents of proteins

The study showed that the contents of proteins from plasma membranes of the roots of tomato seedlings under phosphate starvation approximated to those of the controls （Table 1）. The study also showed that there were no distinct differences of the protein contents from plasma – membrane – depleted vesicles of the roots of tomato seedlings between phosphate starvation treatment and control （Table 1）.

Table 1 Contents of proteins from plasma membranes （PM） and plasma – membrane – depleted vesicles （PDV） of the roots of tomato seedlings

Protein content （μg / g FW）		PM	PDV
	Pi – starved	106.8±16.1	77.5±14.6
	Control	109.2±12.8	82.3±15.3

（The means in Table 1 are from three samples）

Results of SDS – PAGE

The results of SDS – PAGE from plasma membranes of the roots of tomato seedlings

The results showed that， under phosphate starvation for 3 d， there were no remarkable differences of the protein components between phosphate starvation and control except that two polypeptides of 64 kD and 67 kD expressed slightly stronger in phosphate starved seedlings （Fig. 1）.

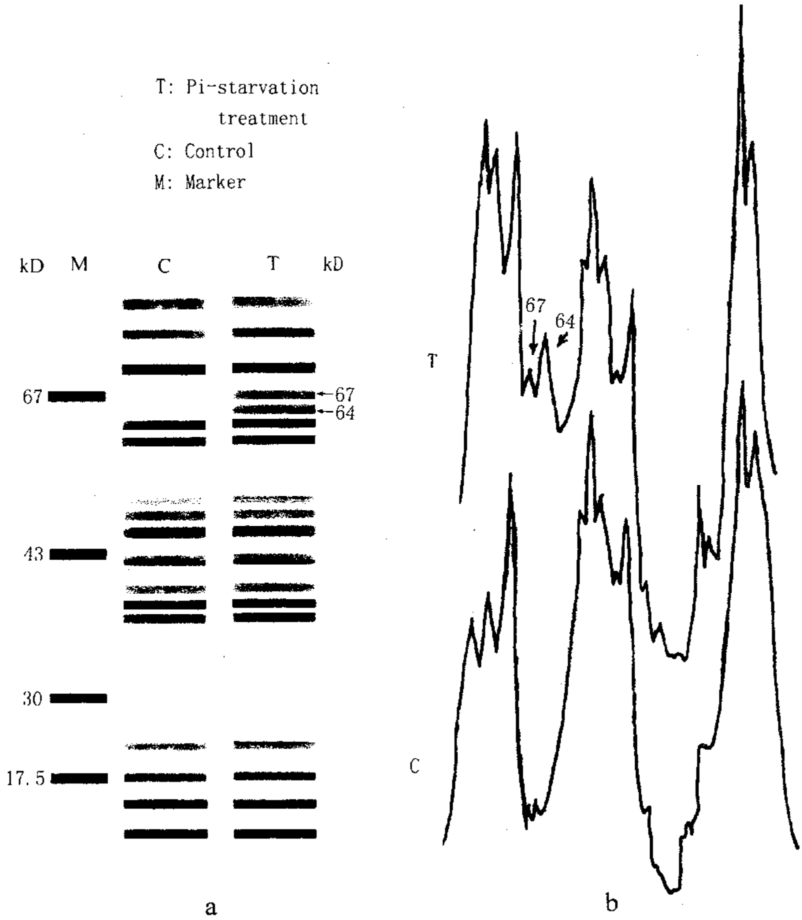

Fig. 1 SDS – PAGE of proteins from plasma membranes of the roots of tomato seedlings under phosphate starvation for 3 d (a: photographs; b: scanning illustrative plates)

The results further showed that, under phosphate starvation for 7 d, changes occurred that four novel polypeptides (mw: 34 kD, 36 kD, 46 kD and 49 kD) appeared in phosphate starved seedlings but not in control (Fig.2). In addition, compared with the synthesis of proteins of the seedlings under phosphate starvation for 3 d (Fig.1), the synthesis of proteins bellow 42 kD was strengthened under phosphate starvation for 7 d (Fig.2). Moreover, CG – PAGE in Fig. 3 showed the same results as those of SDS – PAGE in Fig.2.

The results of SDS – PAGE from plasma – membrane – depleted vesicles of the roots of tomato seedlings

Under phosphate starvation for 3 d and 7 d, the results did not show the obvious differences of the protein components between phosphate starvation treatment and control (Fig.4). In addition, the protein components under phosphate starvation for 7 d were basically identical with those under phosphate

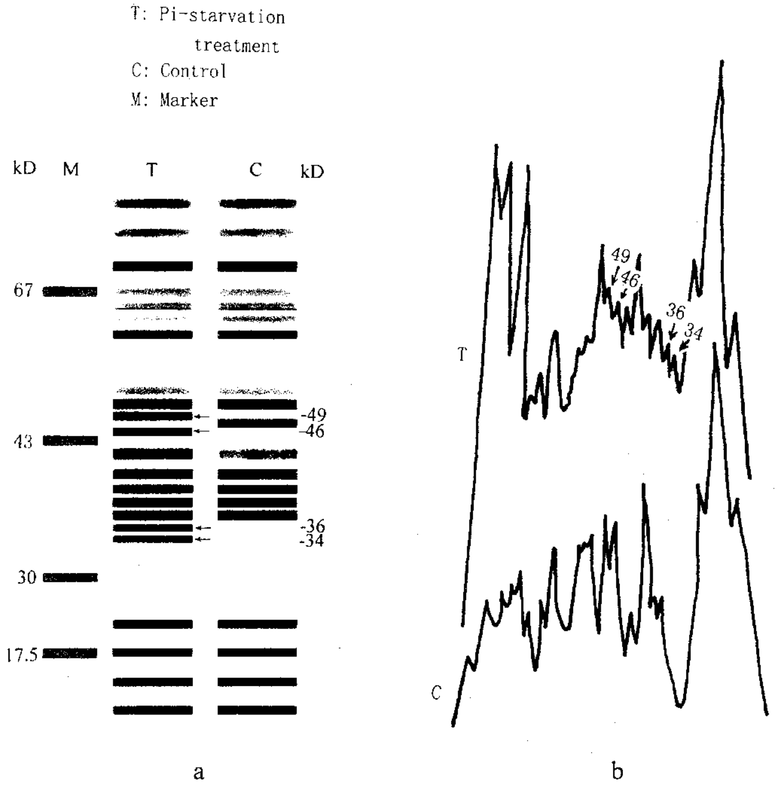

Fig. 2 SDS - PAGE of proteins from plasma membranes of the roots of tomato seedlings under phosphate starvation for 7 d (a : photograph ; b : scanning illustrative plates)

starvation for 3 d (Fig. 4).

Discussion

The results reported here showed that the contents of proteins from plasma membranes of the roots of tomato seedlings under phosphate starvation approximated to those of the controls (Table 1), but the components of polypeptides from plasma membranes under phosphate starvation differed from those of the controls (Fig.2, Fig.3). These results indicated that phosphate stress had no effect on the total amount of synthesis of proteins but had some effect on the differential expression of proteins during protein synthesis.

Although there were no distinct differences of the protein components from plasma membranes between phosphate starvation treatment and control under phosphate starvation for 3 d (Fig.1), there were four polypeptides (34 kD, 36 kD, 46 kD and 49 kD) which appeared obviously in plasma membranes under phosphate starvation for 7 d and not in those of the controls (Fig.2). The results of CG

– PAGE of the plasma membranes further demonstrated that four novel polypeptides above – mentioned were induced by phosphate starvation for 7 d but not possessed by the control (Fig.3), which provided strong support to the results from Fig.2.

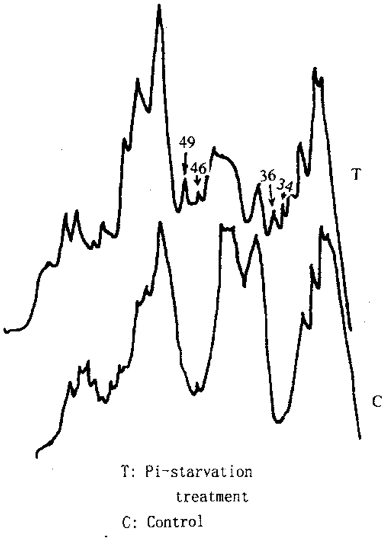

Fig. 3 Scanning illustrative plates of CG – PAGE of proteins from plasma membranes of the roots of tomato seedlings under phosphate starvation for 7 d

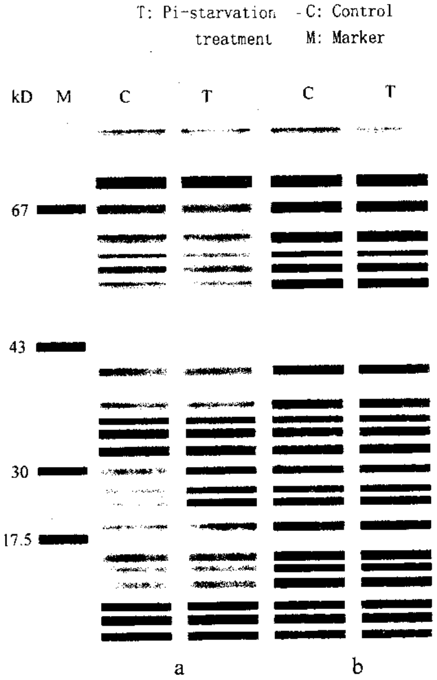

Fig. 4 SDS – PAGE of proteins from plasma – membrane – depleted vesicles of the roots of tomato seedlings under phosphate starvation for 3 d (a) and 7 d (b)

Generally speaking, the universal proteins related to any physiological stress may express in an experiment in which a special process was disturbed by a kind of stress. Because phosphate stress treatment duration in this experiment was relatively short (≤7 d) which had no serious effect on the growth of the seedlings (Song *et al*, 1998), the differential expression of polypeptides above – mentioned (i.e. the polypeptides of 34 kD, 36 kD, 46 kD and 49 kD) may be specific to phosphate stress. Fife *et al* (1990) reported that the polypeptides of 27 kD, 41 kD and 64 kD were newly synthesised in suspension cultured *Brassica nigra* cells under phosphate deprivation. Goldstein et al (1989) found at least 6 novel polypeptides in suspension cultured tomato cells under phosphate starvation and regarded these new polypeptides as the components of phosphate starvation rescue system (PSRS). The PSRS may poly important roles in increasing the capacity of phosphate transport during phsophate starvation. Plants increased the capacity of phosphate transport in response to phosphate stress, which may be an adaptive strategy (Clarkson *et al*, 1991). The increase of the capacity of phosphate transport during phosphate starvation may be the result of regulation of the components of phosphate transporter in translation level. The polypeptides of 34 kD, 36 kD, 46 kD and 49 kD from plasma membranes of the roots of tomato seedlings in our study may be components of phosphate starvation rescue systems induced by phosphate starvation. As a matter of fact, this kind of induction were parallel to derepression of the activities of phosphate transport (or phosphate absorption) under phosphate starvation (Song *et al*, 1998). So we suppose that the novel polypeptides apeared in our experiment might be associated with the changes of phosphate transport under phosphate starvation. However, whether these novel polypeptides are components of phosphate transport proteins is indefinite and the identification of the clear function of these new polypeptides need more expriments such as the further separation, reconstitution and expression of these proteins.

Under phosphate starvation for 3 d, the polypeptides of 64 kD and 67 kD from plasma membranes seemed express a little stronger (Fig. 1), but under phosphate starvation for 7 d, the expression of these two polypeptides seemed have no differences anymore between phosphate starvation and control (Fig. 2). Therefore these two polypeptides may be not the novel specific polypeptides induced by phosphate starvation but reflect the changes in steady – state levels of proteins.

The components of proteins from plasma – membrane – depleted vesicles of the roots of tomato seedlings were also detected in this study. It was supposed that these membrane fractions contained broken mitochondria, nuclear membrane, tonoplast and endoplasmic reticulum membrane (Larsson *et al*, 1987). However, we did not find any distinct differences of these membrane fractions between phosphate stress and control regardless of the stress duration to be 3 d or 7 d (Fig. 4). This result indicated that phosphate starvation, at least short period of that, had no obvious effect on the components of proteins from plasma – membrane – depleted vesicles.

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